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Fructosebisphosphatase Isoenzymes of the Chemoautotroph *Xanthobacter flavus*

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Xanthobacter flavus **employs two fructosebisphosphatase (FBPase)-sedoheptulosebisphosphatase (SBPase) enzymes. One of these is constitutively expressed and has a high FBPase-to-SBPase ratio. The alternative enzyme, which is encoded by *cbbF*, is induced during autotrophic growth. The *cbbF* gene was expressed in *Escherichia coli*, and the FBPase was purified to homogeneity. The purified enzyme has a specific FBPase activity of 114 μmol/min/mg of protein, a Michaelis constant for fructosebisphosphate of 3 μM, and a low FBPase-to-SBPase ratio. CbbF was activated by ATP and inhibited by Ca^{2+}.**

Xanthobacter flavus is a facultative chemoautotrophic bacterium which uses the Calvin cycle for the fixation of CO_2. During autotrophic growth, methanol, formate, or hydrogen is oxidized to provide the cell with energy. The genes encoding the enzymes of the Calvin cycle studied to date are organized into two transcriptional units: the *pgk* gene, encoding phosphoglycerate kinase, and the *cbb* operon (17, 19, 21, 24). The *cbb* operon, which is expressed only during autotrophic growth, encodes the Calvin cycle enzymes ribulosebisphosphate carboxylase (RuBisCO) and phosphoribulokinase. A fructosebisphosphatase (FBPase)-encoding gene (*cbbF*) and a gene with unknown function which is similar to *spoVJ* from *Bacillus subtilis* are also present in this operon (12, 19). The transcription of the *cbb* operon is controlled by CbbR, a LysR-type transcriptional activator (18, 25).

A number of Calvin cycle enzymes, e.g., phosphoglycerate kinase, not only are required for CO_2 fixation but also play an important role during heterotrophic growth. Because of this, it is not surprising that the *pgk* gene is constitutively expressed. Since high-level phosphoglycerate kinase activities are required during autotrophic growth of *X. flavus*, the expression level of the *pgk* gene is increased four- to sixfold after a switch from heterotrophic to autotrophic growth conditions (17). Like phosphoglycerate kinase, FBPase is also required during both heterotrophic and autotrophic growth. However, in contrast to the situation with *pgk*, the only FBPase-encoding gene from *X. flavus* cloned and characterized to date is located within the *cbb* operon, which is not expressed under heterotrophic growth conditions (19, 21). A second FBPase gene expressed in heterotrophically growing cells must therefore be present in *X. flavus.*

In contrast to the situation for the unique Calvin cycle enzymes RuBisCO and phosphoribulokinase, about which detailed information is available, relatively little is known about the other enzymes required for CO_2 fixation. The *cbbF* gene of *X. flavus* has been cloned and characterized previously (14, 19, 21). This paper describes the heterologous expression of this gene in *Escherichia coli* and the subsequent purification and characterization of the FBPase encoded by *cbbF.*

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MATERIALS AND METHODS

Media, growth conditions, and plasmids. The plasmid pWL401 harbors *cbbF* on a 2.0-kb *Bam*HI-*Sal*I fragment downstream from the *lac* promoter of pBluescribt (Vector Cloning Systems, San Diego, Calif.). Plasmid pWL301 contains the *cbbF* gene in the opposite orientation with respect to the *lac* promoter (19). *E. coli* JM101 harboring pWL301 or pWL401 was grown at 37°C on Luria Bertani (LB) medium (22) containing 100 μg of ampicillin per ml. *X. flavus* was grown on minimal medium (15) containing methanol (0.5% [vol/vol]) or succinate (20 mM) as described previously (20). Growth on gluconate (5 mM) followed by addition of formate (20 mM) to induce the *cbb* operon was as described previously (19). The pH of the culture after the addition of formate was kept constant via automatic titration with formic acid (25% [vol/vol]).

DNA manipulations. Plasmid isolation, manipulation of DNA, and Southern hybridizations under stringent conditions (63°C) were done as described previously (25). DNA fragments used as probes in Southern hybridizations were radiolabeled with [^{32}P]dCTP by using the random-primed labeling kit obtained from Boehringer (Mannheim, Germany) as described by the manufacturer. *X. flavus* chromosomal DNA was isolated as described elsewhere (11).

Heterologous expression of *cbbF* in *E. coli*. *E. coli* JM101 harboring pWL301 or pWL401 was grown on LB medium at 37°C (22), diluted into fresh LB medium, and grown until an optical density at 663 nm of 0.5 was reached. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM, and growth was allowed to proceed for an additional 4 h. Cells were harvested via centrifugation, washed once in ice-cold buffer (20 mM Tris-HCl [pH 8.0], 2.5 mM $MgCl_2$, 0.1 mM EDTA) (buffer A), and resuspended in the same buffer.

Purification of heterologously expressed FBPase encoded by *cbbF*. All purification steps were performed at 4°C, except as noted otherwise. Cell extracts of IPTG-induced *E. coli*(pWL401) were freshly prepared by passing the cell suspension twice through a French pressure cell (1.4×10^5 kN/m^2) after the addition of phenylmethanesulfonyl fluoride (0.1 mM). Cell debris was removed by centrifugation at 35,000 × *g* for 30 min. DNase (1 mg/ml) was added to remove DNA from the extract. The cell extract was subjected to an $(NH_4)_2SO_4$ fractionation, in which the FBPase activity was recovered at between 30 and 45% $(NH_4)_2SO_4$ saturation. The FBPase-containing fraction was desalted by using a PD10 column (Pharmacia, Uppsala, Sweden) equilibrated with buffer A. The subsequent steps were performed at room temperature by using a Pharmacia FPLC system (Pharmacia, Uppsala, Sweden). The proteins were loaded on a Mono Q anion-exchange column and eluted with an NaCl gradient (17.5 mM/ml) in buffer A, at a flow rate of 0.5 ml/min. The active fractions were pooled, $(NH_4)_2SO_4$ was added to 1.3 M, and this preparation was loaded on a phenyl-Superose column equilibrated with buffer A containing 1.3 M $(NH_4)_2SO_4$. FBPase was eluted with a decreasing $(NH_4)_2SO_4$ gradient (7.8 mM/ml) in buffer A, at a flow rate of 0.5 ml/min. The active fractions were pooled and desalted by using a PD10 column equilibrated with buffer A, and KCl was added to 1.5 M. This preparation was subsequently loaded on a phenyl-Superose column equilibrated with buffer A containing 1.5 M KCl. FBPase was eluted with a decreasing KCl gradient (37.5 mM/ml) in buffer A, at a flow rate of 1.0 ml/min. Active fractions were pooled and concentrated with an Amicon P30 membrane (Amicon, Danvers, Mass.).

Separation of FBPase activities in *X. flavus* cell extracts. A cell extract of methanol-grown *X. flavus* was passed through a Mono Q column as described above, except that the flow rate was 1.0 ml/min.

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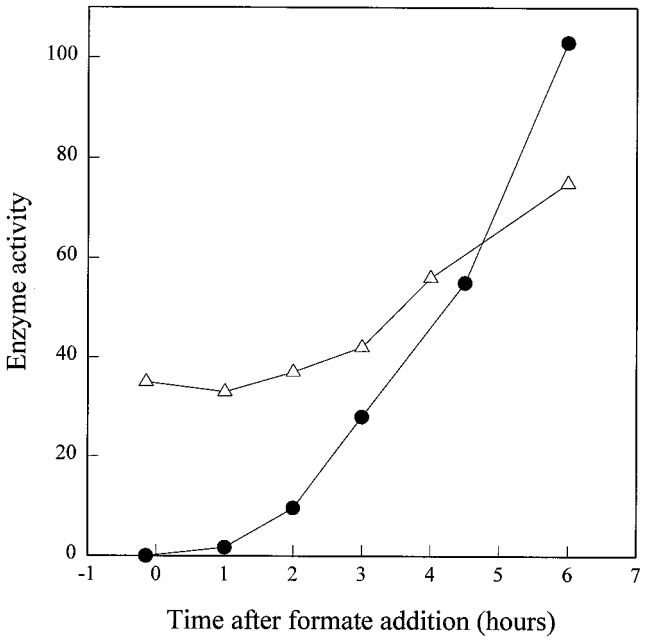

FIG. 1. Enzyme profiles of *X. flavus* growing on 5 mM gluconate. Shown are the results of the addition of 20 mM formate and automatic titration with formic acid (25% [vol/vol] at 0 h). ●, RuBisCO activity; △, FBPase activity. Enzyme activities are in nanomoles per minute per milligram of protein.

Enzyme assays. FBPase activity was determined by using a reaction mixture containing 25 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 150 μg of bovine serum albumin per ml, 1 U of phosphoglucoisomerase, 1 U of glucose 6-phosphate dehydrogenase, 0.4 mM NADP, and 0.5 mM fructosebisphosphate. The fructosebisphosphate-dependent increase in A_{340} as a result of NADP reduction was monitored for at least 3 min at 30°C and was proportional to the amount of extract added. The effect of low-molecular-weight compounds on the FBPase activity was tested by adding these 3 min prior to the addition of fructosebisphosphate. Equimolar amounts of Mg^{2+} were added when ATP was added to the reaction mixture. The FBPase-to-sedoheptulosebisphosphatase (SBPase) activity ratio was determined by the method described by Amachi and Bowien (2), except that 1 mM fructosebisphosphate or sedoheptulosebisphosphate and 50 mM Tris-HCl (pH 8.5) were used. RuBisCO was determined as described elsewhere (7). Protein was determined according to the method of Bradford, with bovine serum albumin as the standard (3).

RESULTS

FBPase activity in *X. flavus*. FBPase is required for both gluconeogenesis and CO_2 fixation via the Calvin cycle. The effects of induction of the Calvin cycle during gluconeogenic growth of *X. flavus* on the activity of FBPase were examined. When formate (20 mM) is added to a culture of *X. flavus* growing on gluconate (5 mM), the activity of FBPase increases 2 h after the addition of formate. A similar increase in activity was previously observed for phosphoglycerate kinase (17). The activity of RuBisCO, indicative of the transcription of the *cbb* operon, which was absent prior to the addition of formate, appeared at the same time (Fig. 1).

***X. flavus* employs two distinct FBPase enzymes during autotrophic growth.** The increased FBPase activity after induction of the Calvin cycle could be due to the expression of *cbbF*, which is located within the *cbb* operon. To examine whether autotrophically grown cells contain an additional FBPase, a cell extract of *X. flavus* grown on methanol was fractionated on a Mono Q anion-exchange column. FBPase was recovered in two activity peaks ($FBPase_I$ and $FBPase_{II}$; approximate ratio, 1.6 to 1), indicating the presence of two FBPase enzymes with different properties (Fig. 2). Further evidence that the two activity peaks represent different enzymes was obtained by comparing the heat stabilities of $FBPase_I$ and $FBPase_{II}$. $FBPase_I$ was inactivated at a higher rate (half-life = 4 min) when incu-

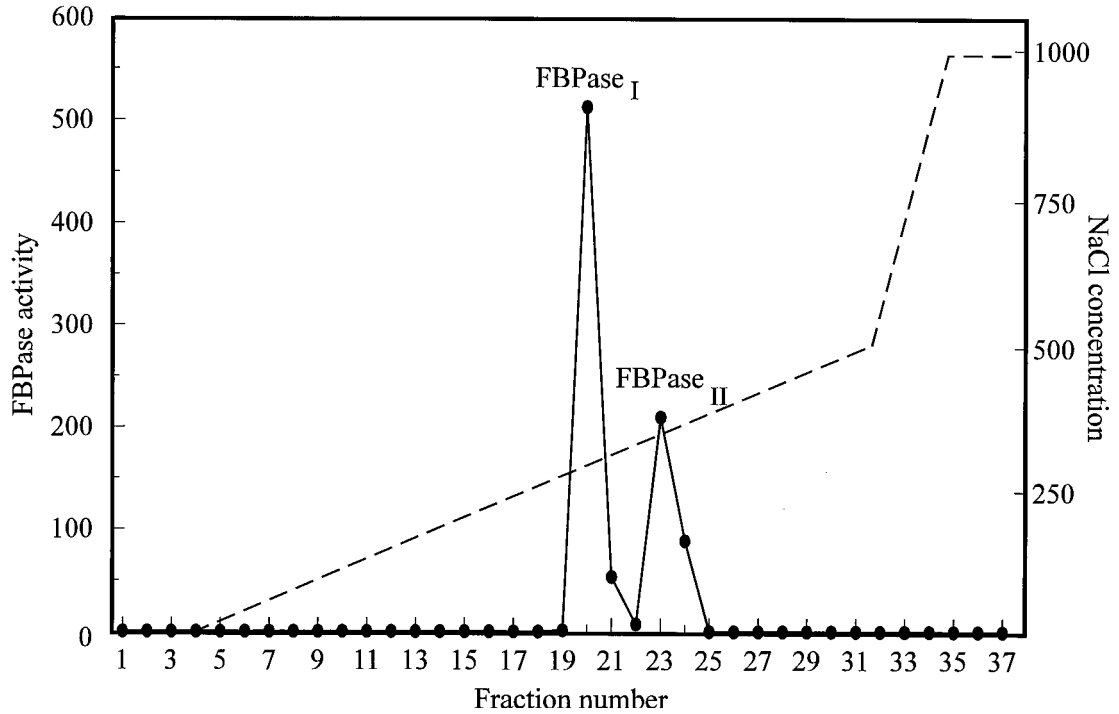

FIG. 2. Separation of FBPase activities on a Mono Q anion-exchange column. The column was loaded with a cell extract of methanol-grown *X. flavus* and eluted with an increasing NaCl gradient (dashed line). FBPase activities (●) are given in nanomoles per minute. The NaCl concentrations shown are millimolar.

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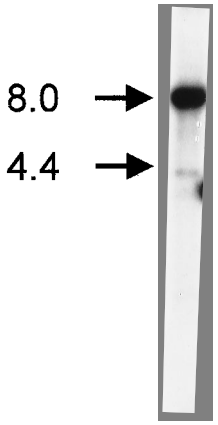

FIG. 3. Southern hybridization of a 1,021-bp *Bgl*II-*Sma*I DNA fragment containing the *cbbF* gene of *X. flavus* to *Eco*RI-digested chromosomal DNA from *X. flavus*. Sizes are indicated in kilobases.

bated at 51°C than $FBPase_{II}$ was (half-life = 15 min). Cell extracts of succinate-grown *X. flavus* contained only the heat-labile form of FBPase, indicating that $FBPase_{II}$ is encoded by *cbbF*, which is specifically induced during autotrophic growth.

In addition to FBPase, SBPase is present in the Calvin cycle. In plants, these are the activities of two distinct enzymes; prokaryotes, however, employ an FBPase with a broad substrate specificity. In *X. flavus*, the FBPase-to-SBPase ratio was 1.0 for $FBPase_I$, whereas the ratio was 0.5 for $FBPase_{II}$. On the basis of these results, we conclude that *X. flavus* possesses two types of FBPase during growth on methanol. The more heat-stabile form of FBPase ($FBPase_{II}$; CbbF) is specifically induced during autotrophic growth and is most likely used to increase the SBPase activity during autotrophic growth of *X. flavus.*

***X. flavus* has two FBPase-encoding genes.** Since the FBPase gene encoded within the *cbb* operon (*cbbF*) is expressed only during autotrophic growth, the FBPase activity observed during heterotrophic growth must be due to an alternative enzyme. This prompted us to investigate whether a second gene encoding FBPase is present on the chromosome of *X. flavus*. A 1,021-bp *Bgl*II-*Sma*I DNA fragment containing the *cbbF* gene of *X. flavus* (21) was radiolabeled and hybridized to chromosomal *X. flavus* DNA digested with *Eco*RI. In addition to a signal for hybridization of the *cbbF* probe to a restriction fragment of the expected size (8 kb) containing the *cbbF* gene, an additional, fainter hybridization signal was consistently observed (Fig. 3). This indicates the presence of a second FBPase-encoding gene on the chromosome of *X. flavus.*

Heterologous expression of *cbbF* encoding $FBPase_{II}$. We previously constructed the plasmids pWL401, on which expression of *cbbF* is under the control of the *lac* promoter, and pWL301, with the *cbbF* gene in the opposite orientation with respect to the *lac* promoter (19). IPTG was added to cultures of *E. coli* JM101 harboring pWL301 or pWL401 to induce transcription from the *lac* promoter. After IPTG addition, FBPase activity in *E. coli*(pWL401) increased rapidly (up to 1.9 μmol/min/mg of protein), whereas it remained undetectable in *E. coli*(pWL301). The appearance of high-level FBPase activities in *E. coli*(pWL401) was accompanied by a decrease in growth rate. Growth of *E. coli*(pWL401) ceased at an optical density at 660 nm of 0.9, whereas growth of *E. coli*(pWL301) was not affected. The dephosphorylation of fructosebisphosphate by FBPase in *E. coli*(pWL401) counteracts the phosphorylation of fructose 6-phosphate, catalyzed by phosphofructokinase during glycolysis. The activity of these enzymes could create a futile cycle which converts ATP to ADP and P_i and may, in part, be responsible for the observed growth inhibition caused by the expression of CbbF in *E. coli.*

TABLE 1. Purification of $FBPase_{II}$ encoded by *cbbF* fromI PTG-induced *E. coli*(pWL401)

Purification step	Sp act[a]	Purification factor	Total protein[b]	Total activity[c]
Cell extract	1.9	1.0	106.5	202.4
$(NH_4)_2SO_4$ fractionation	5.9	3.1	20.5	121.4
Mono Q	26.6	13.6	3.1	85.0
Phenyl-Superose $(NH_4)_2SO_4$	80.0	42.1	1.0	80.1
Phenyl-Superose KCl	114.0	60.0	0.7	76.1

[a] Micromoles per minute per milligram of protein.
[b] Milligrams.
[c] Micromoles per minute.

Purification of CbbF. FBPase present in IPTG-induced *E. coli* (pWL401) was purified in four steps (Table 1) and was found to be homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4). The purified FBPase was stable, with virtually no loss of activity when stored at −20°C for 2 weeks in buffer A. The FBPase subunit had a molecular mass of 40 kDa, which is in close agreement with the mass (38,738 Da) predicted from the CbbF amino acid sequence (21). The SBPase activity of the purified FBPase was twice as high as the FBPase activity. The specific activity of the purified FBPase was 114 μmol/min/mg of protein, and the Michaelis constant for fructosebisphosphate was 3 μM. Similar values have been reported for FBPase enzymes from other sources (13, 27, 28). The activity of FBPase was strictly dependent on the presence of Mg^{2+}.

Various compounds were tested for their ability to stimulate or inhibit FBPase activity. In general, FBPase enzymes participating in gluconeogenesis are inhibited by AMP, whereas those functioning in the Calvin cycle are not sensitive to AMP (1, 2). The activity of the *cbbF*-encoded FBPase was not affected by AMP (1.0 mM). Addition of ATP (1.0 mM) to the reaction mixture resulted in a twofold increase in FBPase activity. In contrast, the FBPase activity in cell extracts of succinate-grown *X. flavus* ($FBPase_I$; see above) was not stimulated by ATP. The chloroplast FBPase is inhibited by Ca^{2+}, which

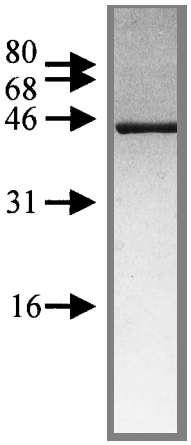

FIG. 4. Coomassie brilliant blue-stained denaturing polyacrylamide gel showing FBPase purified from IPTG-induced *E. coli* transformed with the *cbbF* expression vector pWL401. Numbers refer to molecular weight standards and are in thousands.

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may function to inhibit this enzyme when the chloroplast is not illuminated (5). The *cbbF*-encoded FBPase is also strongly inhibited by Ca^{2+}, with a K_i of 67 μM.

DISCUSSION

The induction of the *cbb* operon via the addition of formate to gluconate-grown *X. flavus* resulted in a simultaneous increase of both FBPase and RuBisCO. Increased activities of Calvin cycle enzymes other than RuBisCO or phosphoribulokinase are required to support the high rate of CO_2 fixation via the Calvin cycle. This is exemplified by an *X. flavus* mutant which contains a defective *pgk* gene. This mutation virtually abolished the activity of this phosphoglycerate kinase and rendered the mutant unable to grow autotrophically (17). In sharp contrast, the growth rate of the *pgk* mutant on succinate decreased by only 13%, which reflects the fact that the gluconeogenic requirement of growing cells accounts for only 5% of the total catabolic and anabolic needs (10).

The location of *cbbF* in the *cbb* operon causes it to be expressed only under autotrophic growth conditions. Since FBPase activity is also required during heterotrophic growth, a second gene must be present. The results from the Southern hybridization experiments and the separation of FBPase activities in cell extracts of heterotrophically and autotrophically grown cells show that this is indeed the case. Since all facultatively autotrophic bacteria examined to date contain a *cbbF* gene in the *cbb* operon, the use of two FBPase enzymes is probably widespread (6, 8, 23, 26). In the gram-positive autotrophic bacterium *Nocardia opaca*, constitutive and inducible FBPases have been shown to be present (2).

Unlike plants, in which the SBPase and FBPase reactions are catalyzed by two different enzymes, *X. flavus* employs an FBPase with a broad substrate specificity. The *cbbF*-encoded enzyme has in fact an SBPase activity twofold higher than its FBPase activity. This enzyme may therefore be used primarily to increase the SBPase activity, which is essential during autotrophic but not heterotrophic growth.

The FBPase encoded by *cbbF* has a number of properties in common with the chloroplast FBPase. The FBPases from both organisms are activated when the cell or chloroplast is energized, although the activation mechanisms are different. The chloroplast, but not the cytosolic, plant FBPase is activated by light via a thioredoxin mechanism (4, 9, 13, 16), whereas the autotrophic FBPase from *X. flavus* is stimulated by ATP. Stimulation of the autotrophic FBPase by ATP or light is not unexpected, since the Calvin cycle has to function maximally under conditions of carbon starvation and energy surplus. The FBPase from *Alcaligenes eutrophus* and the autotrophic FBPase from *Nocardia opaca* are inhibited by ATP, which is rather surprising (1, 2). Both the chloroplast FBPase and the autotrophic FBPase from *X. flavus* are inhibited by Ca^{2+}. It has been suggested that the Ca^{2+} inhibition of the chloroplast FBPase is a means to block the Calvin cycle when Ca^{2+} is released into the chloroplast when this organelle is no longer energized via illumination (5). It is unclear whether the Ca^{2+} inhibition of the *X. flavus* enzyme has such a specific physiological function.

From the data presented in this paper, it becomes clear that the FBPase encoded by *cbbF*, which is specifically induced during autotrophic growth conditions, is a specialized enzyme uniquely adapted for its role in the Calvin cycle. Apparently, a specialized phosphoglycerate kinase is not required during autotrophic growth of *X. flavus*, since this enzyme is encoded by the constitutively expressed *pgk* gene located outside the *cbb* operon (10). The use of a specialized FBPase during autotrophic growth is most likely common among facultative autotrophic bacteria, since the *cbb* operons of these bacteria all contain a *cbbF* gene (6, 8, 23, 26).

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